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Research Article

Simultaneous determination of lappaconitine hydrobromide and isopropiram fumarate in rabbit plasma by capillary electrophoresis with electrochemiluminescence detection

A CE electrochemiluminescence (CE–ECL) method for simultaneous determination of lappaconitine hydrobromide (LH) and isopropiram fumarate (IF) has been first established, with a chemically modified platinum electrode by europium (III)-doped Prussian blue analogue film as a working electrode. The conditions for CE separation and ECL detection are discussed and optimized in detail. It has been proved that 20 mmol/L phosphate buffer (pH 8.5) containing 5% (v/v) ACN and 0.17 mol/L SDS could achieve the most favorable resolution, and the high sensitivity of detection was obtained by maintaining the detection potential at 1.23 V. Under optimized conditions, a baseline separation for the two analytes was achieved within 6 min, and the standard curves were linear in the range of $1.0\times10^{-7} \sim 5.0 \times 10^{-5}$ g/mL for LH and $4.0 \times 10^{-8} \sim 1.0 \times 10^{-5}$ g/mL for IF with the detection limits (3σ) of 6.6×10^{-8} g/mL for LH and 3.7×10^{-8} g/mL for IF, respectively. The precisions of intra- and interday measurements for LH and IF were less than 4.21 and 2.61%, respectively. The applicability of the proposed method was illustrated in the determination of LH and IF in rabbit plasma with recoveries between 95.6 and 103.0%.

Keywords:
Capillary electrophoresis / Electrochemiluminescence / Isopropiram fumarate / Lappaconitine hydrobromide / Plasma DOI 10.1002/elps.201100630

1 Introduction

Lappaconitine hydrobromide (LH) and isopropiram fumarate (IF) (Fig. 1) are analgesic and anti-inflammatory drugs, which are the most effective drugs presently available for the treatment of malignant tumor and other intractable pain [1, 2]. It is important to develop sensitive and reliable methods for the determination of LH and IF in biological fluids to enable their clinical applications to achieve optimum therapeutic effects and minimize side effects. But it is a pity that there are only a few studies for LH and IF detection, including spectrophotometry [3] and HPLC [4, 5].

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Abbreviations: CL, chemiluminescence; **CV**, cyclic voltammogram; **ECL**, electrochemiluminescence; **Eu-PB/Pt electrode**, platinum microelectrode modified with europium (III)-doped Prussian blue analogue film; **IF**, isopropiram fumarate; **LH**, lappaconitine hydrobromide

CE have received considerable interest in analytical chemistry recently due to their promising features such as relatively short analysis time, high resolution, and minute consumption of samples and reagents [6, 7]. A high-performance capillary electrophoresis (HPCE) approach was employed to identify mixtures of the toxic alkaloids including LH with a UV detector [8]. Chemiluminescence (CL), including electrochemiluminescence (ECL) analysis often offers lower background noise, inherent higher detection sensitivity, and has become a candidate for a new and sensitive CE detection scheme. Especially, CE combining with ECL (CE–ECL) based on tris(2,2′-bipyridyl) ruthenium (II) ($Ru(bpy)_3^{2+}$), as an efficient and sensitive analytical technique, has been extensively applied for the determination of analytes containing alkyl amines group [9–11]. However, as far as we know, there are no reports on the application of CE–ECL assay to quantitative analysis of LH or IF.

In this paper, an efficient and sensitive CE–ECL method has been first established for the simultaneous determination of LH and IF in rabbit plasma. A platinum microelectrode modified with europium (III)-doped Prussian blue analogue film (Eu-PB/Pt) was prepared and applied as the working electrode in order to significantly improve the reliability and sensitivity of the method.

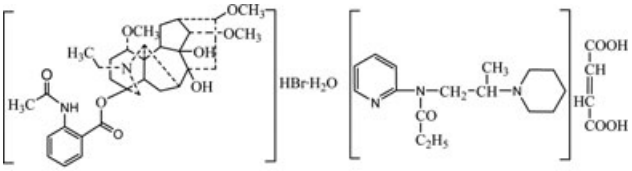

Figure 1. Molecular structures of LH and IF.

2 Materials and methods

2.1 Reagents and chemicals

Reagents and chemicals used were all of analytical reagent grade. Tris(2,2'-bipyridyl) ruthenium(II) dichloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$) was obtained from Aldrich (Milwaukee, WI, USA) and used without further purification. Reference substance of LH and IF was purchased from the Chinese Pharmaceutical and Biological Test Institute (Beijing, China) and freshly prepared with water under lightproof conditions just before use. Stock solutions of $Ru(bpy)_3^{2+}$ and working standard solutions were stored at 4°C in a refrigerator and filtered through a 0.22 μm membrane prior to injection.

2.2 Apparatus

A MPI-A CE–ECL system was bought from Xi'an Remax Electronic and Science Technological Co. (Xi'an, China). A CHI832 electrochemical analyzer (Shanghai Chenhua Apparatus Corporation, Shanghai, China) was used for modification of the platinum-working electrode. Double-distilled water (≥18.2 MΩ) was prepared by Milli-Q water purification system (Millipore, Bedford, MA, USA). The end-column ECL detection was composed of a three-electrode system with a Eu-PB/Pt disk working electrode ($\Phi = 0.5$ mm), a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode (KCl saturated). A 50 cm length of uncoated fused-silica capillary (50 μm id, Yongnian Optical Fiber Factory, Hebei, China) was used for separation.

2.3 Electrophoresis conditions

The schematic diagram of CE–ECL system and preparation of Eu-PB/Pt electrode have been described previously [12]. The detection reservoir was filled with 5 mM $Ru(bpy)_3^{2+}$ and 80 mM phosphate buffer (pH 8.0) before analysis and replaced every 3 h to eliminate depletion effect. Running buffer solution was 20 mM phosphate buffer (pH 8.5) containing 5% (v/v) ACN and 0.17 mol/L SDS. During the experiment, a separation voltage of 16 kV was applied across the capillary and a detection potential of 1.23 V was applied at the working electrode. After the baseline of ECL signal reached a constant value, the electrokinetic injection was used for sample introduction at 10 kV for 10 s, and the electropherogram was recorded. The voltage of PMT for collecting the ECL signal was set at −800 V in the process of detection. The inlet end of the capillary was held at a positive potential, and the outlet end was maintained at ground. The distance between the working electrode and the outlet of the capillary was set at approximately 150 μm with the aid of an optical microscope. At the beginning of each day, the capillary was necessary to be flushed with 0.1 M NaOH for 3 min, double-distilled water for 3 min, and then equilibrated with the running buffer for 5 min so as to maintain an active and reproducible inner surface.

2.4 Sample preparation

Plasma for method development and validation was obtained from rabbit, which was provided by School of Basic Medical Sciences, Lanzhou University. Blood sample of 10 mL was collected, following administration, immediately heparinized, and centrifuged for 10 min at 3500 rpm in order to separate the plasma sample in upper layer. Then, the plasma sample was stored at −20°C until assay. For plasma extraction, the following procedure was performed. A 0.4 mL plasma sample and 2.0 mL ethyl acetate were pipetted into a clean centrifugation tube, and then the mixture was made alkaline with 0.05 mL of 0.1 M NaOH. The tube was placed in a mechanical shaker for 10 min and subsequently centrifuged at 3500 rpm for 10 min. The organic layer was transferred into another tube and the procedure was repeated twice. At last, the combined organic layers were evaporated to dryness under a stream of dry nitrogen at 40°C. The dry residue was dissolved with 0.05 mL of 0.1 M NaOH and diluted with water to 50 mL, then pass through a 0.22 μm membrane and being directly injected into the capillary electrophoresis system and analyzed.

3 Results and discussion

3.1 Effect of the Eu-PB/Pt electrode

The electro-oxidation characterization of $Ru(bpy)_3^{2+}$ was tested before and after modification of the working electrode by cyclic voltammetry. It was shown that the peak current from the electro-oxidation of $Ru(bpy)_3^{2+}$ was enhanced significantly at the Eu-PB/Pt electrode in comparison with the current response at the bare platinum electrode, as shown in Fig. 2A (curves a and b). As a result, more $Ru(bpy)_3^{3+}$ was produced in the modified electrode and then it was reduced by the analytes, resulting in more production of excited state of $Ru(bpy)_3^{2+}$. Consequently, an enhanced ECL signal of $Ru(bpy)_3^{2+}$ could be expected in the prepared electrode

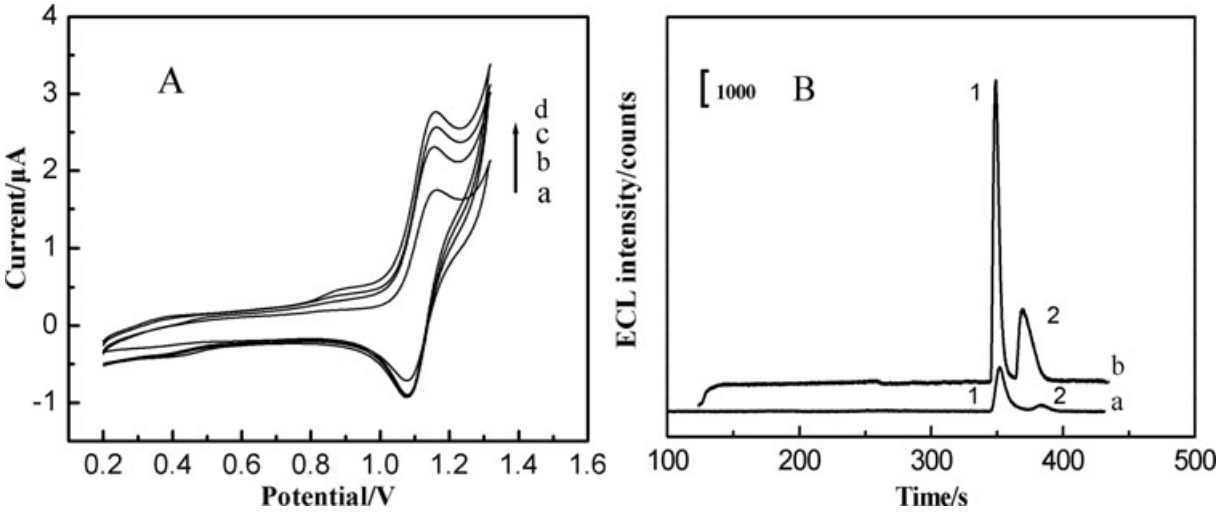

Figure 2. (A) Cyclic voltammograms of 5 mmol/L $Ru(bpy)_3^{2+}$ in 80 mmol/L phosphate buffer (pH 8.0) (a) at bare Pt electrode; (b) at Eu-PB/Pt electrode; (c) 1.0×10^{-5} g/mL LH was added in the above $Ru(bpy)_3^{2+}$ solution at Eu-PB/Pt electrode; (d) 6.0×10^{-7} g/mL IF was added in the above $Ru(bpy)_3^{2+}$ solution at Eu-PB/Pt electrode. Scan rate, 100 mV/s. (B) Electropherograms of 1.0×10^{-5} g/mL LH and 6.0×10^{-7} g/mL IF standard solution: (a) at bare Pt electrode; (b) at Eu-PB/Pt electrode. Separation capillary, 50 μm id, 50 cm length; sample injection, 10 s at 10 kV; separation voltage, 16 kV; detection potential, 1.23 V; running buffer, 20 mmol/L phosphate buffer (pH 8.5) containing 5% (v/v) ACN and 0.17 mol/L SDS. 1. IF; 2. LH.

when the excited state $Ru(bpy)_3^{2+}$ returned to the ground state $Ru(bpy)_3^{2+}$.

Further, ECL responses of the two analytes, LH and IF, were investigated by using a constant detection potential mode. As illustrated in Fig. 2B, the ECL signals of the two analytes in the prepared electrode were both seven times higher than that in a bare platinum electrode. The result indicated that the use of the Eu-PB/Pt working electrode benefited from the improved sensitivity for analytes in $Ru(bpy)_3^{2+}$-based ECL system.

In addition, it was found that the prepared electrode helped to improve the stability of the method and to obtain available CE–ECL signals for analytes in plasma samples mainly due to reduction of poisoning effect of complex matrices. On the other hand, the modified electrode was also stable enough for repetitive use in the detection system within 1 month with no need for electrode replacement. Consequently, the prepared electrode provided significant improvement in the sensitivity and reliability of the method.

3.2 CE–ECL behavior of the analytes

The cyclic voltammograms (CVs) of $Ru(bpy)_3^{2+}$, LH, and IF were compared at Eu-PB/Pt working electrode in order to discuss the effect of the two analytes on $Ru(bpy)_3^{2+}$-based ECL process. As shown in Fig. 2A (curves b, c, and d), oxidation currents increased when LH and IF were added in the $Ru(bpy)_3^{2+}$ solution. The above trend of CVs was similar to that of some analytes at bare platinum electrode, indicating that the analytes with tertiary amines group were oxidized by $Ru(bpy)_3^{3+}$ in the detection cell and consequently more excited state of $Ru(bpy)_3^{2+}$ was produced [13]. Thus, emission light intensity related to ruthenium species was enhanced by LH and IF. Given this, the possible ECL mechanism at the Eu-PB/Pt working electrode could be expressed as follows, as similar to that at an unmodified electrode [14]:

$$Ru(bpy)_3^{2+} - e \xrightarrow{\text{electrocatalytic oxidation}} Ru(bpy)_3^{3+} \quad (1)$$

$$LH - e \longrightarrow LH^{*+} \quad (2)$$

$$LH^{*+} \longrightarrow LH^{*} + H^{+} \quad (3)$$

$$LH^{*} + Ru(bpy)_3^{3+} \longrightarrow Ru(bpy)_3^{2+*} + \text{products} \quad (4)$$

$$Ru(bpy)_3^{2+*} \longrightarrow Ru(bpy)_3^{2+} + h\nu \quad (5)$$

On the other hand, ECL response and CE behavior of the two analytes, IF and LH, have been compared in the work. As seen in Fig. 2B, the ECL response was more sensitive to IF than to LH. Generally, the ECL intensity for alkyl amines followed the order of tertiary > secondary > primary. So, the luminescence mainly caused by the tertiary amine group in the structure of IF and LH. Thus, the possible reasons could be contributed to the following points that related to the nitrogen

atom of tertiary amine group. First, substituent attached to the α- or β-carbon atom bonding with nitrogen atom has great effect on the ECL intensity [15, 16]. An electron-withdrawing substituent (ether group) attached to the β-carbon atom of nitrogen atom in the structure of LH, decreasing the stability of positive nitrogen radical ion (–N^+·–), and hence leading to a weaker ECL intensity. On the other hand, ECL intensity was also affected by dimensional conformation surrounding nitrogen atom [17]. The planar conformation of IF decreased the ionization energy of nitrogen atom, stabilized the positive nitrogen radical ion, and helped to increase the ECL intensity of the compound. Contrarily, the perpendicular conformation of LH increased dimensional block, interfered with stability of positive nitrogen radical ion, and therefore decreased ECL intensity.

Besides, it was also found in Fig. 2B that the migration time of IF is shorter than that of LH. It is considered in CE–ECL detection that the separation of analytes mainly depends on the different migration rates in the field, and substantially depends on the difference of their electric charges and particle sizes. Generally speaking, the more charges the ions carried, the more thorough dissociation, the smaller ions size, and the faster electrophoresis rate. Given this, electrophoresis rate of LH was slower due to a larger molecular size and a less thorough dissociation from dimensional block.

3.3 Optimization of system

3.3.1 Effect of detection potential

Detection potential on the Eu-PB/Pt electrode has great effect on the ECL intensity. The results showed that at lower potentials, a very weak ECL response was obtained. Starting at approximately 1.15 V, the increase in ECL signal became readily apparent. The intensity curves for LH and IF both reached the highest value and showed the most favorable detection potential at 1.23 V (vs. Ag/AgCl), as seen in Fig. 3. The reason was contributed to the increased production of $Ru(bpy)_3^{3+}$ with a rise of potential since excited state of $Ru(bpy)_3^{2+}$ was still regarded as the luminescent intermediate on the Eu-PB/Pt electrode, as mentioned above. When the potential exceeded 1.23 V, the ECL response weakened slightly because the oxidation of water and other impurities on the working electrode prevented the generation of electro-produce $Ru(bpy)_3^{3+}$ [18]. Hence, the potential of 1.23 V was then applied in the following experiments for the detection of LH and IF.

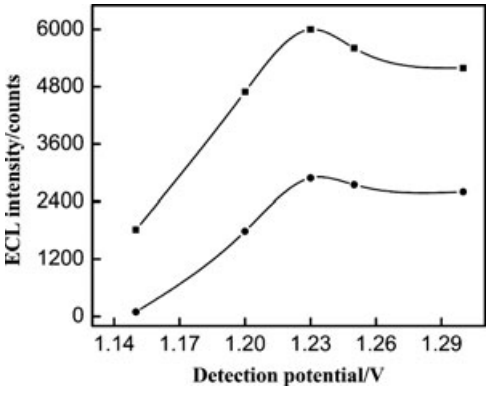

Figure 3. Effect of detection potential on ECL intensity: (■)IF, (●)LH. Other conditions are the same as in Fig. 2.

3.3.2 Effect of running buffer

Separation and determination of LH and IF were studied in different buffer systems including phosphate, acetate, Tris-HCl, citric acid–sodium citrate, and borate in different pH ranges. It was found that acetate, Tris-HCl, and citric acid–sodium citrate exhibited unstable signals under alkaline conditions, where higher ECL responses could be obtained for the analytes. Further, the effects of phosphate and borate were studied and phosphate buffer was finally chosen in terms of the more stable baseline and signals, shorter analysis time, and higher efficient separation with better peak shape. Also, the pH effect of phosphate on electrophoresis separation and ECL sensitivity were investigated in a wide pH range of 4.0 ~ 9.5 at intervals of 0.5 pH units. As illustrated in Fig. 4A, the resolution (R) of LH and IF reached the maximum at pH 8.5 and then decreased with the increase in pH value. Here R was calculated using the following equation: $R = 2(t_{R2} - t_{R1})/(W_1 + W_2)$, where t_{R1} and t_{R2} were the migration times of two adjacent analytes and W_1 and W_2 were the peak widths of two adjacent analytes measured at the baseline. At the same time, it was also been found in Fig. 4A that the ECL intensities for both IF and LH almost reached the maximum value at pH 7.5 ~ 8.5. When the pH was higher than these values, the ECL intensity decreased slightly. The possible reason was considered as the reduced availability of $Ru(bpy)_3^{2+}$ at high pH due to the competitive reaction of OH^- which consumed considerable $Ru(bpy)_3^{3+}$ [19]. Considering a good separation result and a high ECL intensity, the optimal pH was 8.5. Further, when the pH of phosphate was fixed, the effect of concentration of phosphate was studied when it was adjusted from 5 to 30 mmol/L. It was shown in Fig. 4B that running buffer working at a high phosphate concentration allowed improving the resolution between LH and IF, but above 20 mmol/L, noise increased gradually, baseline shifted, and ECL intensity decreased due to excessive heating caused by Joule effect. Hence, 20 mmol/L phosphate was chosen as the running buffer.

3.3.3 Effect of organic additives

The effect of methanol, ethanol, ACN, and SDS in the buffer on the separation and detection has been tested in order to obtain better separation efficiency but not to lose sensitivity. The results showed that the addition of methanol and ethanol

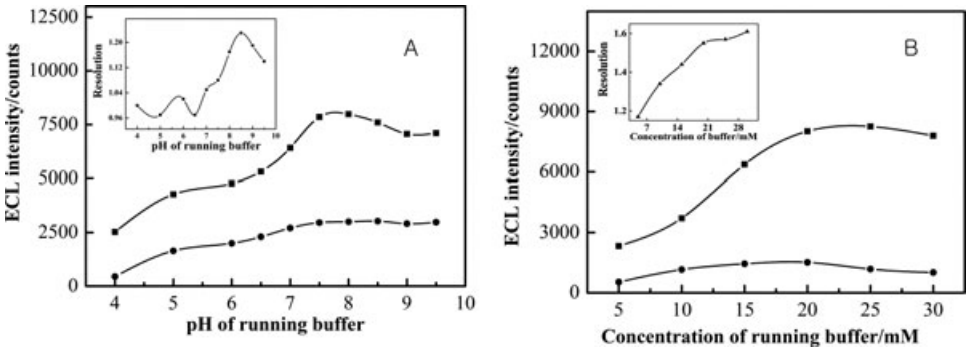

Figure 4. (A) pH effect of running buffer on CE–ECL detection; (B) effect of running buffer concentration on CE–ECL detection. (■)IF, (•)LH. Other conditions are the same as in Fig. 2.

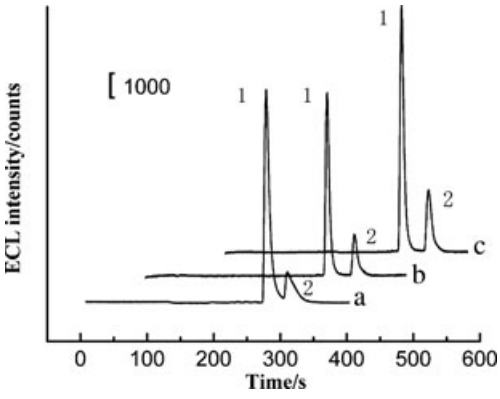

Figure 5. Electropherograms of IF and LH. Running buffer, (a) 20 mmol/L NaH_2PO_4-Na_2HPO_4 solution (pH 8.5); (b) added 5% (v/v) ACN to (a) solution; (c) added 5% (v/v) ACN and 0.17 mol/L SDS to (a) solution. Other conditions are the same as in Fig. 2. 1. IF; 2. LH.

had little effect on separation of the two analytes; while the addition of 5% (v/v) ACN solution could significantly improve the resolution and the peak shape. However, the addition of ACN itself decreased ECL intensity for the two analytes. Hence, 0.17 mol/L SDS was also added into the phosphate buffer to increase the luminescence, as shown in Fig. 5.

3.3.4 Effect of separation voltage

It was necessary to investigate the separation voltage because it drove samples through the capillary and impacted on ECL intensity and analysis time. More analytes arrived in the diffusion layer of working electrode within a given time since the EOF increased with the increasing separation voltage, making for a higher ECL signal. In addition, analysis time became shorter with increasing separation voltage. However, the strong flow of effluent from the capillary might reduce the concentration of $Ru(bpy)_3^{3+}$ at the electrode surface, thereby, reducing the efficiency of light producing reaction [20]. Thus, after comprehensive consideration for the ECL intensity and analysis time, the best choice for separation voltage was 16 kV.

3.3.5 Effect of injection voltage and injection time

The effect of the injection voltage and injection time on the ECL intensities of LH and IF were studied. On the one hand, at higher injection voltage or larger injection time, more analytes appeared at the working electrode, which produced a higher ECL signal. On the other hand, too much analytes could not react at the electrode immediately and diffuse back into the solution. Hence, overloading effect might take place, the ECL peaks were usually broadened and the plate number decreased [21]. Thus, by weighing a higher ECL intensity and the improved column efficiency, 10 kV and 10 s were compromised as the optimal injection voltage and injection time, respectively.

3.3.6 Effect of capillary to electrode distance

The effect of the distance between the working electrode and the outlet of the capillary on ECL signals was studied. It was found that the highest ECL response for the analytes was obtained when the distance was set at ca. 150 μm. Too small a capillary to electrode distance (ca. 70 μm) resulted in a great decrease in ECL peak because the concentration of $Ru(bpy)_3^{3+}$ near the working electrode was diluted by CE buffer. While too large a distance (ca. 220 μm) also led to the decreased ECL signal with the decrease of mass transport of analytes into the detection region [22]. Thus, the capillary to electrode distance was selected at ca. 150 μm.

3.4 Linearity, detection limit, and precision

Under the optimum conditions established above, the calibration graphs for LH and IF concentration versus ECL intensity were linear at a certain concentration range, respectively. And the fitted correlation coefficients were all exceeded 0.9997, as listed in Table 1. The detection limits of 6.6×10^{-8} g/mL for LH and 3.7×10^{-8} g/mL for IF were obtained at the signal to noise ratio of 3 (S/N = 3), respectively, which were both less than those obtained by methods mentioned above [3–5].

The precision of the proposed method was validated by reduplicate injections ($n = 5$) of a mixed standard solution

Table 1. Linearity and detection limit of the method

Compound	Linear range (g/mL)	Regression equation	Correlation coefficient	Detection limit (g/mL)
LH	$1.0 \times 10^{-7} \sim 5.0 \times 10^{-6}$	$\Delta I = -0.01 + 2.29 \times 10^{8}C$	0.9998	6.6×10^{-8}
	$5.0 \times 10^{-6} \sim 5.0 \times 10^{-5}$	$\Delta I = 676.37 + 1.00 \times 10^{8}C$	0.9999	
IF	$4.0 \times 10^{-8} \sim 5.0 \times 10^{-7}$	$\Delta I = -607.09 + 1.66 \times 10^{10}C$	0.9999	3.7×10^{-8}
	$5.0 \times 10^{-7} \sim 1.0 \times 10^{-5}$	$\Delta I = 4568.24 + 5.99 \times 10^{9}C$	0.9997	

containing 1.0×10^{-5} g/mL LH and 6.0×10^{-7} g/mL IF. RSDs of the ECL peak heights of LH and IF were 2.61 and 1.29% within a day, and 4.21 and 2.32% in 3 days, respectively. The RSDs of the migration times of LH and IF were 0.41 and 0.39% within a day, and 0.90 and 0.75% in 3 days, respectively.

3.5 Applications

To evaluate the applicability of the present CE–ECL method to real samples, it has been used to determine LH and IF in rabbit plasma. Rabbit plasma samples were pretreated according to the procedure described above. The typical electropherograms of blank plasma sample and the plasma samples spiked with 10 μg/mL LH and 0.6 μg /mL IF were illustrated in Fig. 6. The results showed that LH and IF could be well separated in plasma samples and the two analytes were not detected in blank plasma sample but could be seen and resolved in plasma samples spiked with standard substances. Finally, the recoveries of 103.0% for LH and 95.6% for IF were obtained, respectively, as seen in Table 2.

4 Concluding remarks

A new analytical procedure based on CE–ECL was introduced to detect LH and IF in rabbit plasma for the first time. LH and IF acted as a coreactant to enhance $Ru(bpy)_3^{2+}$-based ECL intensity on an Eu-PB/Pt electrode. The proposed method took advantages of rapidity, high-sensitivity, wide linear range, and good accuracy, and showed good prospects with respect to their performances in the biological and clinical fields.

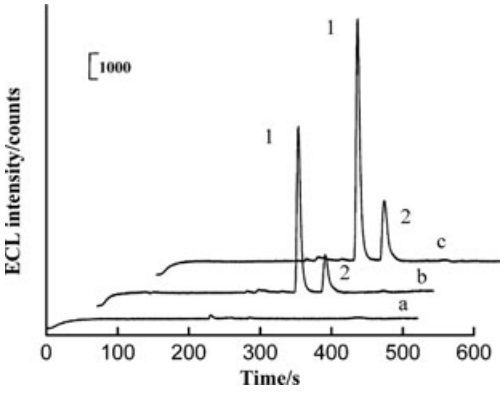

Figure 6. Electropherograms of blank plasma sample (a), spiked plasma sample with 10 μg /mL LH and 0.6 μg /mL IF (b), and with 20 μg /mL LH and 0.9 μg /mL IF (c). Other conditions are the same as in Fig. 2.

Table 2. Results for the determination of LH and IF in rabbit plasma

Compound	Found (μg/mL)	RSD(%) ($n = 5$)	Added (μg/mL)	Recovered (μg/mL)	Recovery (%)
LH	9.8	2.91	10	20.4	103.0
IF	0.61	1.53	0.3	0.87	95.6

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The authors have declared no conflict of interest.

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